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Molecular mechanism(s) of action of norepinephrine on the expression of the angiotensinogen gene in opossum kidney cells

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Molecular mechanism(s) of action of norepinephrine on the expression of the angiotensinogen gene in opossum kidney cells.

Background. Norepinephrine (NE) is the major endogenous neurotransmitter of the renal sympathetic nerves interacting with both the α- and β-adrenoceptors in the renal proximal tubules. We have previously reported that isoproterenol and iodoclonidine stimulate the expression of the angiotensinogen (ANG) gene in opossum kidney (OK) proximal tubular cells via the β_1-adrenoceptor and α_2-adrenoceptor, respectively. We hypothesized that NE may interact with the β- and/or α_2-adrenoceptors to stimulate the expression of the ANG gene in OK cells.

Methods. The fusion genes containing the various lengths of the 5′-flanking regulatory sequence of the rat ANG gene fused with a human growth hormone (hGH) gene as a reporter were stably transfected into the OK cells. The stimulatory effect of NE on the expression of the fusion genes was evaluated by the amount of immunoreactive hGH (IR-hGH) secreted into the culture medium.

Results. The addition of NE stimulated the expression of the fusion gene, pOGH (ANG N-1498/+18) in a dose-dependent manner. The stimulatory effect of NE was inhibited in the presence of propranolol, atenolol, Rp-cAMP, yohimbine, staurosporine, H-7 and U73122 but not in the presence of ICI 118,551 and prazosin. The addition of a combination of isoproterenol and iodoclonidine synergistically stimulated the expression of pOGH (ANG N-1498/+18) as compared to the addition of isoproterenol and iodoclonidine alone. Furthermore, the addition of NE, forskolin, 8-Br-cAMP or phorbol 12-myristate (PMA) stimulated the expression of pOGH (ANG N-806/-779/-53/+18), a fusion gene containing the putative cAMP responsive element (CRE, ANG N-806/-779) upstream of the ANG promoter (ANG N-53/+18) in OK 95 cells, but had no effect on the expression of fusion genes containing the mutant of the CRE.

Conclusion. These studies demonstrate that the stimulatory effect of NE on the expression of the ANG gene in OK cells may be mediated via both the β_1- and α_2-adrenoceptors and via the CRE (ANG N-806/-779) in the 5′flanking region of rat ANG gene.

The existence of an intrarenal renin-angiotensin system (RAS) has now been generally accepted [1, 2]. Angiotensinogen (ANG) mRNA has been localized in rat proximal tubules by the techniques of *in situ* hybridization [3] and polymerase chain reaction (PCR) [4]. Recent studies have also shown that the mRNA components of RAS, including ANG, renin, angiotensin-converting enzyme and angiotensin II receptor (AT_1-receptor) are expressed in murine (mouse and rat) immortalized proximal tubular cell lines [5, 6]. We [7] as well as Ingelfinger et al [8] have also demonstrated that the ANG mRNA is expressed in opossum kidney (OK) proximal tubular cells. Thus, these studies demonstrate that the intrarenal angiotensin II (Ang II) is probably derived from the ANG that is synthesized by the renal proximal tubular cells.

We have previously reported that isoproterenol and iodoclonidine stimulate the expression of the ANG gene in OK cells [9, 10]. The effect of isoproterenol is mediated via the β_1-adrenoceptor and cAMP-dependent protein kinase A (PKA) pathway [9], whereas the effect of iodoclonidine is mediated via the α_2-adrenoceptor and protein kinase C (PKC) pathway [10]. Our studies confirm the reports of Nakamura and Johns [11] that low levels of renal nerve stimulation increase the ANG mRNA levels in the rat kidney *in vivo*. Furthermore, our studies [9, 10] and those of Nakamura and Johns [11] together indicate the presence of a functional relationship between the renal sympathetic nervous system and the activation of local intrarenal RAS. Thus, the local formation of renal Ang II may play an important role in the physiology of the renal proximal tubular cells (that is, sodium and fluid reabsorption) [12–15].

Norepinephrine (NE) is the major endogenous neurotransmitter of the renal sympathetic nerves [16, 17] and it interacts with both the α- and β-adrenoceptors in the renal proximal tubules [18–21]. Thus, the objective of the present studies was to investigate whether NE modulates the expression of the ANG gene in OK cells. Our studies showed that addition of NE stimulates the expression of the ANG gene via both β_1-and α_2-adrenoceptors and their respective PKA and PKC pathways. Moreover, we demonstrated that the effect of NE on the expression of the ANG

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gene is mediated via the putative cAMP-responsive element (CRE) in the 5′-flanking region of the ANG gene.

METHODS

Materials

The plasmid, pRSV-Neo, containing the coding sequence for Neomycin (Neo) with the Rous Sarcoma Virus (RSV) enhancer/promoter sequence fused in the 5′-end of the Neomycin gene was a gift from Dr. Teresa Wang (Dept. of Pathology, Stanford University, Stanford, CA, USA). The plasmid, pTKGH, containing the thymidine kinase (TK) enhancer/promoter sequence fused to the 5′-end of the hGH gene was purchased from the Nichols Institute of Diagnostics (La Jolla, CA, USA).

The radioimmunoassay kit for hGH (RIA-hGH) was a gift from NIADDK (NIH, Bethesda, MD, USA). The RIA procedure has been described previously [22]. NIAMDD-hGH-I-1 (AFP-4793 B) was used for both iodination and as a hormone standard. The limit of sensitivity of the assay was 0.1 ng/ml. The inter- and intra-assay coefficients of variation were 10% ($N = 10$) and 12% ($N = 10$), respectively.

Yohimbine hydrochloride (α_2-adrenoceptor antagonist), P-iodoclonidine hydrochloride (α_2-adrenoceptor agonist), prazosin hydrochloride (α_1-adrenoceptor antagonist), R(−)-isoproterenol (+)-bitartrate salt (β-adrenoceptor agonist), atenolol (β_1-adrenoceptor antagonist), ICI 118,551 (β_2-adrenoceptor antagonist), phorbol 12-myristate 13-acetate (PMA, a stimulator of protein kinase C), staurosporine (an inhibitor of protein kinase C), H-7 (an inhibitor of protein kinase C), U73122 (an inhibitor of phospholipase C and A_2), S(−)-propranolol hydrochloride (an inhibitor of β_1- and β_2-adrenoceptors), 8-bromo-cAMP, forskolin, Rp-cAMP (an inhibitor of the cAMP-dependent protein kinase A I and II) were all purchased from Research Biochemicals Inc (RBI, Natick, MA, USA). (−)Arterenol bitartrate (norepinephrine) was purchased from Sigma Chemicals (St. Louis, MO, USA).

Oligonucleotides for the putative CRE of the rat ANG gene (ANG-CRE), N-806 to N-779 (5′ AAG AGA TTA CTT GAC GTA CTG GAT GCA A 3′) [22], mutant 1 (M1) (5′ AAG AGA TTA CTT GAC TTA CTG GAT GCA A 3′), mutant 2 (M2) (5′ AAG AGA TTA CTT GAA TTA CTG GAT GCA A 3′) and mutant 3 (M3) (5′ AAG AGA TTA CTT ATA TTA CTG GAT GCA A 3′) were synthesized by Biosynthesis, Inc. (Lewisville, TX, USA).

$Na^{125}I$ was purchased from Dupont, New England Nuclear (NEN, Boston, MA, USA). Calcium chloride was purchased from Mallinckrodt, Inc. (Montreal, Quebec, Canada); Geneticin (G 418) was purchased from Bethesda Research Laboratories (Gibco-BRL, Burlington, Ontario, Canada). Other molecular biology grade reagents were obtained either from Sigma Chemicals, Gibco-BRL, Boehringer-Mannheim (Dorval, Quebec, Canada), Pharmacia Inc. (Baie d'Urfe, Quebec, Canada) or Promega-Fisher, Inc. (Montreal, Quebec, Canada).

Construction of fusion genes

The method of construction of the ANG-GH fusion genes, pOGH (ANG N-1498/+18), pOGH (ANG N-960/+18), pOGH (ANG N-688/+18), pOGH (ANG N-280/+18) and pOGH (ANG N-53/+18) has been described previously [22]. To construct the fusion genes, pOGH (ANG N-806/-779/-53/+18), and its mutants (that is, M_1, M_2, and M_3), the double-strand DNA fragment (ANG N-806/-779) with the *Hin*dIII enzyme restriction site on both the 5′ and 3′-ends was inserted upstream of the minimal promoter of rat ANG gene (ANG N-53/+18) in the pOGH (ANG N-53/+18) [22] that had been previously digested with the restriction enzyme *Hin*dIII and alkaline phophatase.

The sequence and orientation for the fusion genes were confirmed by dideoxy sequencing with SP6 primer (Promega-Fisher, Inc.) and restriction enzyme digestion mapping.

Cell culture

The opossum kidney (OK) proximal tubular cell line was obtained from the American Tissue Culture Collection (ATCC; Rockville, MD, USA). This cell line is derived from the kidney of a female American opossum and retains several properties of proximal tubular epithelial cells in culture [23, 24] and expresses a low level of ANG mRNA [7, 8]. The culture conditions of the OK cells have been described previously [9, 10, 25].

OK cell stable transformants

OK 27, OK 960, OK 688, OK 280, OK 53 and OK 13 cells are stable transformants with pOGH (ANG N-1498/+18), pOGH (ANG N-960/+18), pOGH (ANG N-688/+18), pOGH (ANG N-280/+18), pOGH (ANG N-53/+18) and pTKGH integrated into OK cellular genomes, respectively. The method of obtaining these transformants had been previously reported [9, 10]. Briefly, the ANG-GH fusion gene and the plasmid pRSV-Neo were co-transfected (20 mg) each into OK cells utilizing calcium phosphate-mediated endocytosis. The stable transformants were selected by growing the cells in the presence of G418 (Geneticin; Gibco, Inc.). OK 95, OK 95/M1, OK 95/M2, OK 95/M3 cells are stable transformants with pOGH (ANG N-806/-779/N-53/+18) or its mutants and pRSV-Neo fusion genes co-integrated into OK cellular genomes. The method for the selection of OK cell stable transformants with the high expression of the fusion gene was identical to the method described previously for OK 27 cells [9, 10].

Effect of norepinephrine on the expression of the ANG-GH fusion gene in OK cell stable transformants

OK cell stable transformants were plated at a density of 1 to 2 $\times$ 10^5 cells/well in six-well plates and incubated

overnight in DMEM containing 10% FBS. Then, cell growth was arrested by incubation in serum-free medium for 24 hours. Subsequently, various concentrations of NE (10^{-13} to 10^{-5} M) were added to the culture medium containing 1% resin and charcoal-treated FBS and incubated for 24 hours. At the end of the incubation period, media were collected and kept at −20°C until assayed for IR-hGH.

To compare the inhibitory effect of propranolol, atenolol, ICI 118,551, yohimbine, prazosin, Rp-cAMP, staurosporine, H-7 and U73122 on the expression of ANG-GH fusion gene in OK cell transformants, various concentrations (10^{-13} to 10^{-7} M) of the antagonists or inhibitors were added in the presence of NE (10^{-9} M) for 24 hours. At the end of the incubation period, media were collected and kept at −20°C until assay for IR-hGH.

To compare the effect of NE on the expression of various fusion genes in OK 960, OK 688, OK 280, OK 53 and OK 13 cells, NE (10^{-9} M) was added to the culture medium containing 1% resin and charcoal-treated FBS and incubated for 24 hours. At the end of the incubation period, media were collected and kept at −20°C until assayed for IR-hGH.

The resin and charcoal-treated FBS was prepared by incubation with 1% activated charcoal and 1% AG 1 × 8 ion-exchange resin (Bio-Rad Laboratories, Richmond, CA, USA) for 16 hours or more at room temperature as described by Samuels, Stanley and Shapiro [26]. This procedure removed endogenous steroid and thyroid hormones from the FBS as demonstrated by Samuels et al [26].

Statistical analysis

The experiments were performed at least three to four times in triplicate. The data were analyzed with Student's *t*-test or analysis of variance (ANOVA). A probability level of $P \leq 0.05$ was regarded as significant.

RESULTS

Effect of norepinephrine on the expression of ANG-GH fusion genes in OK cell stable transformants

Figure 1 shows the expression of the pOGH (ANG N-1498/+18) in OK 27 cells in the presence of various concentrations (10^{-13} to 10^{-5} M) of NE. A dose-dependent relationship between NE concentrations and the stimulation of expression of pOGH (ANG N-1498/+18) was observed for NE at 10^{-13} M to 10^{-7} M. The maximal stimulation of expression of the pOGH (ANG N-1498/+18) was found with 10^{-9} M to 10^{-7} M for NE, whereas the addition of concentrations greater than 10^{-7} M (that is, 10^{-5} M) of norepinephrine had no effect.

Figure 2 shows the effect of NE (10^{-9} M) or isoproterenol (10^{-9} M) or iodoclonidine (10^{-9} M) on the expression of the pOGH (ANG N-1498/+18) in OK 27 cells without (Fig. 2A) or with (Fig. 2B) the pre-incubation with a high concentration of NE (10^{-5} M) for 24 hours. It is apparent that the pre-incubation of OK 27 cells with NE (10^{-5} M) abolished the stimulatory effect of NE, isoproterenol or iodoclonidine on the expression of the pOGH (ANG N-1498/+18; Fig. 2B). These studies suggest that the pre-incubation with high concentrations of NE (10^{-5} M) will desensitize or down-regulate the β- and α_2-adrenoceptors in OK 27 cells.

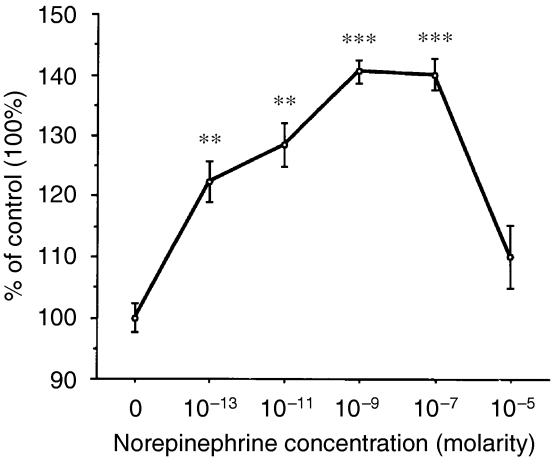

Fig. 1. Effect of norepinephrine on the expression of pOGH (ANG N-1498/+18) in OK 27 cells. Cells were incubated for up to 24 hours in the presence of various concentrations of norepinephrine (10^{-13} to 10^{-5} M). Media were harvested after 24 hours of incubation and assayed for immunoreactive human growth hormone (IR-hGH). The concentrations of IR-hGH in the absence of norepinephrine represents the control level (that is, 1.65 ± 0.03 ng/ml of IR-hGH). Each point represents the mean ± SD of three determinations (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from three other experiements.

Figure 3 shows that addition of either propranolol or yohimbine inhibits the stimulatory effect of NE on the expression of the pOGH (ANG N-1498/+18) in OK 27 cells in a dose-dependent manner. The effective dose for the inhibition of the stimulated expression (by norepinephrine) of the pOGH (ANG N-1498/+18) was found with 10^{-11} M of propranolol ($P \leq 0.05$) and 10^{-9} M yohimbine ($P \leq 0.05$). Yohimbine or propranolol at 10^{-7} M completely inhibited the stimulatory effect of NE ($P \leq 0.01$). These studies suggest that the stimulatory effect of NE is mediated via both β_1-adrenoceptor and α_2-adrenoceptor.

The inhibitory effect of various adrenoceptor antagonists on the expression of the pOGH (ANG N-1498/+18) in OK 27 cells stimulated by NE (10^{-9} M) is shown in Figure 4. Propranolol, atenolol and yohimbine at 10^{-7} M completely inhibited the stimulatory effect of NE ($P \leq 0.01$), whereas ICI 118,551 and prazosin had no effect. The studies further confirm that the effect of NE is mediated via both β_1-adrenoceptor and α_2-adrenoceptor.

Figure 5 shows that addition of Rp-cAMP, staurosporine or U73122 inhibits the stimulatory effect of NE on the expression of pOGH (ANG N-1498/+18) in OK 27 cells in a dose-dependent manner. The maximal and half-maximal inhibition of the stimulated expression of the pOGH (ANG

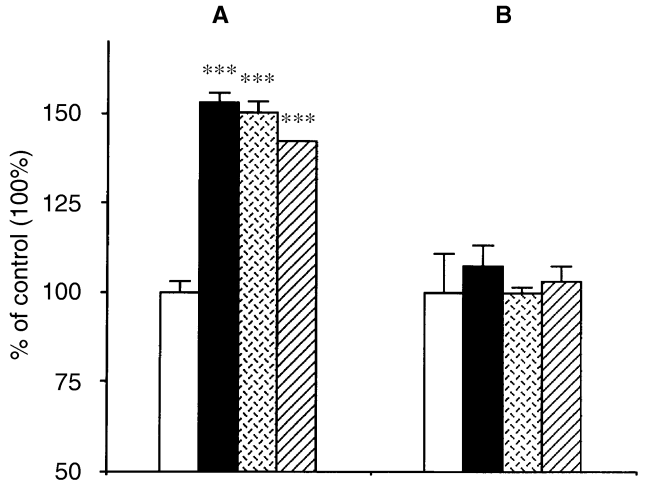

Fig. 2. Effect of norepinephrine, isoproterenol and iodoclonidine on the expression of the pOGH (ANG N-1498/+18) in OK 27 cells when the cells were pre-incubated with a high level of norepinephrine. Cells were pre-incubated for 24 hours without (*A*) or with (*B*) 10^{-5} M norepinephrine. Then the media were replaced with the fresh media containing 10^{-9} M norephinephrine or 10^{-9} M isoproterenol or 10^{-9} M iodoclonidine and incubated further for 24 hours. Subsequently, the media were harvested and assayed for IR-hGH. The concentration of IR-hGH in the medium without the addition of drugs in A or B (that is, 2.5 ± 0.1 ng/ml or 4.0 ± 0.4 ng/ml) is expressed as 100% (control). Each point represents the mean ± SD of three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Symbols are: (□) control medium without the addition of drugs; (■) medium in the presence of 10^{-9} M norepinephrine; (▨) medium in the presence of 10^{-9} M isoproterenol; (▨) medium in the presence of 10^{-9} M iodoclonidine. Similar results were obtained from two other experiments.

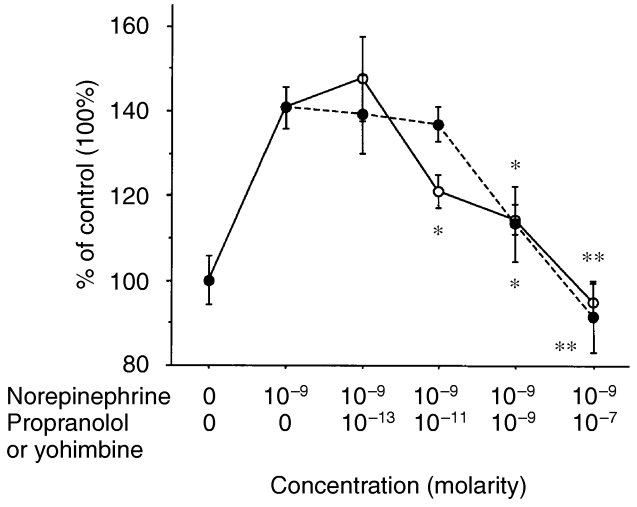

Fig. 3. Inhibitory effect of propranolol (β-adrenoceptor antagonist) or yohimbine (α_2-adrenoceptor antagonist) on the expression of pOGH (ANG N-1498/+18) in OK 27 cells stimulated by norepinephrine. The cells were incubated for up to 24 hours in the presence of norepinephrine (10^{-9} M) and various concentrations of propranolol (10^{-13} to 10^{-7} M) or yohimbine (10^{-13} to 10^{-7} M). Media were harvested and assayed for the level of IR-hGH. The concentration of IR-hGH in the absence of NE or propranolol or yohimbine represents the control level (1.53 ± 0.06 ng/ml of IR-hGH). The inhibitory effect of propranolol or yohimbine was compared to cells that were incubated with 10^{-9} M norepinephrine. Results are expressed as the mean ± SD of three determinations (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Symbols are: (○) propranolol; (●) yohimbine. Experiments were repeated three times.

N-1498/+18) was found with 10^{-7} M ($P \leq 0.01$) and 10^{-11} M ($P \leq 0.05$) Rp-cAMP. These studies indicate that the cAMP-dependent protein kinase AI and II is involved in the expression of the fusion gene stimulated by NE. Similarly, the maximal and half-maximal inhibition of the stimulated expression of the pOGH (ANG N-1498/+18) was found with 10^{-7} M ($P \leq 0.05$) and 10^{-11} M ($P \leq 0.01$) staurosporine or U73122, respectively. The addition of H-7 (an inhibitor of PKC) also inhibited the stimulatory effect of NE on the expression of pOGH (ANG N-1498/+18) in OK 27 cells in a dose-dependent manner (Fig. 6). The maximal and half-maximal inhibition of the stimulated expression of the pOGH (ANG N-1498/+18) was found with 10^{-7} M ($P \leq 0.01$) and 10^{-9} M ($P \leq 0.05$), respectively. These studies indicate that protein kinase C may also mediate the effect of NE on the expression of the ANG gene in OK cells.

Effect of a combination of both iodoclonidine and isoproterenol on the expression of pOGH (ANG N-1498/+18) in OK 27 cells

Figure 7 shows that the stimulatory effect of NE on the expression of pOGH (ANG N-1498/+18) was similar to the addition of isoproterenol or iodoclonidine alone. The addition of a combination of both isoproterenol and iodoclonidine, however, significantly enhanced the expression of the pOGH (ANG N-1498/+18; that is, 230%) compared to the addition of isoproterenol (145%) or iodoclonidine (146%) alone ($P \leq 0.05$). These studies indicate that there is probably a synergistic effect of both β_1- and α_2-adrenoceptors on the expression of pOGH (ANG N-1498/+18) in OK 27 cells.

Effect of NE on the expression of ANG-GH fusion genes and pTKGH in OK cells

Figure 8 shows that the addition of NE (10^{-9} M) stimulated the expression of pOGH (ANG N-1498/+18), pOGH (ANG N-960/+18), pOGH (ANG N-688/+18) in OK 27, OK 960 and OK 688 cells compared to the controls (that is, without the addition of NE), respectively. The addition of NE had no stimulatory effect on the expression of pOGH (ANG N-280/+18), pOGH (ANG N-53/+18) and pTKGH in OK 280, OK 53 and OK 13 cells compared to the controls, respectively.

Effect of NE on the expression of pOGH (ANG N-806/-779/-53/+18) in OK 95 cells

Figure 9 shows that the presence of propranolol or yohimbine inhibited the stimulatory effect of NE on the expression of pOGH (ANG N-806/-779/-53/+18) in OK 95 cells in a dose-dependent manner. The maximal and half-maximal inhibition of the stimulated expression of the

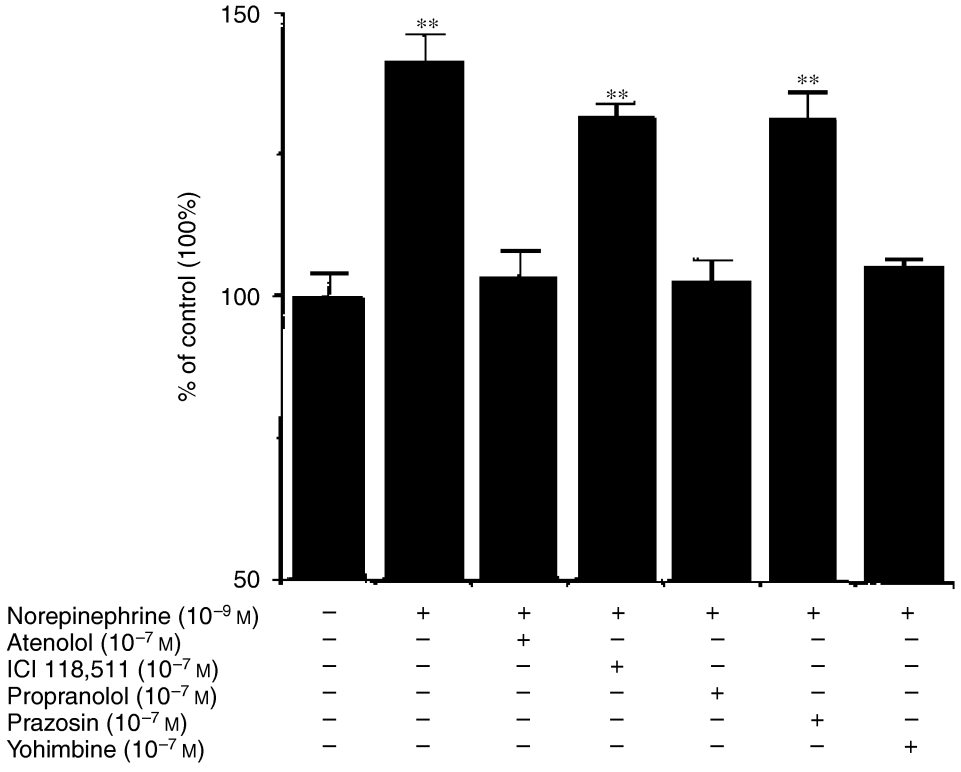

Fig. 4. Inhibitory effect of adrenoceptor antagonists on the expression of pOGH (ANG N-1498/+18) in OK 27 cells stimulated by norepinephrine (10^{-9} M). OK 27 cells were incubated for up to 24 hours in the presence of norepinephrine (10^{-9} M) and 10^{-7} M of various adrenoceptor antagonists. Media were harvested and assayed for the level of IR-hGH. The concentration of IR-hGH in the absence of norepinephrine or adrenoceptor antagonists is the control level (that is, 1.45 ± 0.06 ng/ml of IR-hGH). The inhibitory effect of adrenoceptor antagonists was compared to cells that were incubated with 10^{-9} M norepinephrine. Results were expressed as the mean ± SD of three determinations (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from two other experiments.

pOGH (ANG N-806/-779/-53/+18) was found with 10^{-7} M and 10^{-11} M propranolol or yohimbine, respectively. These studies indicate that the effect of NE on the expression of pOGH (ANG N-806/-779/-53/+18) is mediated via both β_1- and α_2-adrenoceptors, respectively.

Figure 10 shows the result of the addition of 8-bromo-cAMP, forskolin and PMA on the expression of the pOGH (ANG N-806/-779/-53/+18) in OK 95 cells. The addition of 8-bromo-cAMP (10^{-3} M), forskolin (10^{-9} M) or PMA (10^{-9} M) significantly stimulated the expression of pOGH (ANG N-806/-779/-53/+18) in OK 95 cells compared to the controls (that is, absence of 8-bromo-cAMP, forskolin or PMA). Since the DNA fragment, ANG N-806/-779 contains the DNA sequence, TGACGTAC (N-795 to N-788) which is very similar to the consensus CRE (that is, TGACGTCA), these studies indicate that the DNA fragment, ANG N-806/-779, is probably the functional CRE of the rat ANG gene.

Figure 11 shows that the addition of Rp-cAMP (10^{-7} M), U73122 (10^{-7} M) or staurosporine (10^{-7} M) also inhibited the stimulatory effect of NE on the expression of pOGH (ANG N-806/-779/-53/+18) in OK 95 cells. These studies demonstrate that the stimulatory effect of NE on the expression of pOGH (ANG N-806/-779/-53/+18) in OK 95 cells is mediated via both PKA and PKC signal transduction pathway.

Figure 12 shows that the addition of NE (10^{-9} M), 8-Br-cAMP (10^{-3} M) or PMA (10^{-7} M) had no stimulatory effect on the expression of the mutants (that is, M1, M2 and M3) of pOGH (ANG N-806/-779/-53/+18) in OK 95/M1, OK 95/M2 and OK 95/M3 cells compared to the control (without the addition of NE, forskolin or PMA). These studies demonstrate that the DNA sequence, TGACGTAC (N-795/-788) is the motif of the cAMP-responsive element (CRE; that is, ANG N-806/-779) of the rat ANG gene.

Effect of NE or forskolin or 8-Br-cAMP or PMA on the expression of pOGH (ANG N-53/+18) in OK 53 cells

Figure 13 shows that addition of NE (10^{-9} M) or forskolin (10^{-7} M) or 8-Br-cAMP (10^{-3} M) or PMA (10^{-9} M) had no stimulatory effect on the expression of pOGH (ANG N-53/+18) in OK 53 cells. These studies indicate that the minimal promoter (ANG N-53 to N+18) of the rat ANG gene is not sufficient to respond to the addition of NE or PKA or PKC signal transduction pathways.

DISCUSSION

Our present studies showed that addition of NE alone directly stimulated the expression of pOGH (ANG N-1498/

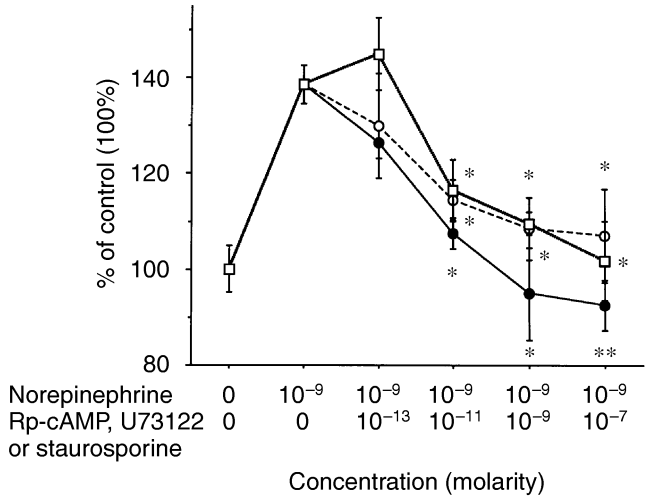

Fig. 5. Inhibitory effect of Rp-cAMP or staurosporine or U73122 on the expression of pOGH (ANG N-1498/+18) in OK 27 cells stimulated by norepinephrine. Cells were incubated for up to 24 hours in the presence of norepinephrine (10^{-9} M) and various concentrations of Rp-cAMP (10^{-13} to 10^{-7} M) or staurosporine (10^{-13} to 10^{-7} M) or U73122 (10^{-13} to 10^{-7} M). Media were harvested and assayed for the level of IR-hGH. The concentration of IR-hGH in the absence of NE or Rp-cAMP or staurosporine or U73122 is the control level (that is, 2.25 ± 0.06 ng/ml of IR-hGH). The inhibitory effect of Rp-cAMP or staurosporine or U73122 was compared to cells that were incubated with 10^{-9} M norepinephrine. Each point represents the mean ± SD of three determinations ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.005$). Symbols are: (●) Rp-cAMP; (○) staurosporine; (□) U73122. Similar results were obtained from two other experiments.

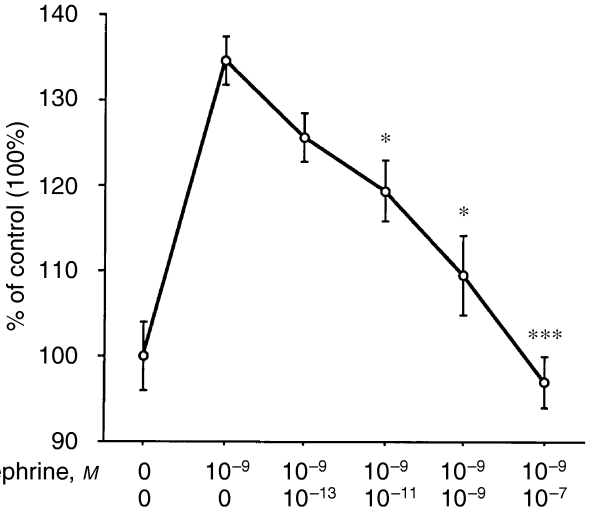

Fig. 6. Inhibitory effect of H-7 on the expression of pOGH (ANG N-1498/+18) in OK 27 cells stimulated by norepinephrine. The cells were incubated for 24 hours in the presence of norepinephrine (10^{-9} M) and various concentrations of H-7 (10^{-13} to 10^{-7} M). Media were harvested and assayed for the level of IR-hGH. The concentration of IR-hGH in the absence of NE represents the control level (that is, 3.14 ± 0.2 ng/ml of IR-hGH). The inhibitory effect of H-7 was compared to cells which were incubated with 10^{-9} M norepinephrine. Results are expressed as the mean ± SD of three determinations ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.005$). Similar results were obtained from another experiment.

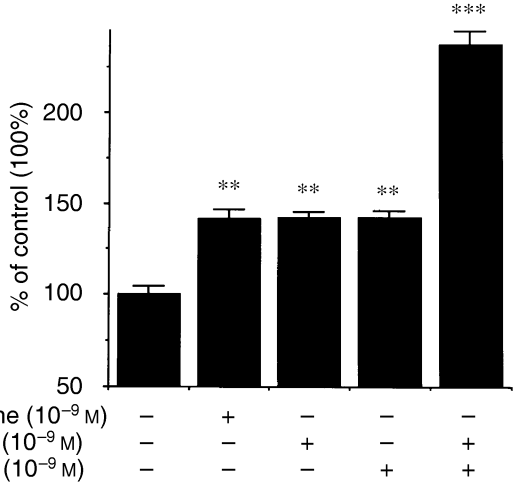

Fig. 7. Effect of a combination of both isoproterenol and iodoclonidine on the expression of pOGH (ANG N-1498/+18) in OK 27 cells. Cells were incubated for up to 24 hours in the presence of NE (10^{-9} M) or isoproterenol (10^{-9}) or iodoclonidine (10^{-9} M) or a combination of both isoproterenol (10^{-9} M) and iodoclonidine (10^{-9} M). Media were harvested after 24 hours of incubation and assayed for IR-hGH. The concentration of IR-hGH in the absence of NE or adrenoceptor agonists represents the control level (that is, 3.79 ± 0.05 ng/ml of IR-hGH). Each point represents the mean ± SD of three determinations ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.005$). Similar results were obtained from two other experiments.

+18) in OK 27 cells in a dose-dependent manner (that is, 10^{-13} to 10^{-7} M; Fig. 1). At present, we do not understand why higher concentrations of NE (that is, 10^{-5} M) had no stimulatory effects on the expression of the pOGH (Ang N-1498/+18) in OK 27 cells. One possible explanation may be that the exposure of OK cells to high levels of NE may desensitize or downregulate its own adrenoceptors. Indeed, our studies (Fig. 2B) showed that the pre-incubation of OK 27 cells with 10^{-5} M NE abolished the stimulatory effect of NE, isoproterenol and iodoclonidine, supporting the notion that the β- and α-adrenoceptors are subject to desensitization by high levels of NE. Furthermore, these results are supported by the observations of Suzuki et al [27] and Kurose and Lefkowitz [28] that β- and α-adrenoceptors are subject to desensitization by their own agonists. Obviously, more experiments along these lines are warranted.

The stimulatory effect of NE was inhibited by the presence of propranolol or yohimbine (Fig. 3) as well as by the presence of atenolol, but not ICI 118,511 and prazosin (Fig. 4). These studies demonstrate that the effect of NE may be mediated via both β_1-adrenoceptor and α_2-adrenoceptor. These studies confirm our previous studies that the activation of the β-adrenoceptor or α_2-adrenoceptor alone stimulated the expression of pOGH (ANG N-1498/+18) in OK 27 cells [9, 10].

The addition of Rp-cAMP, staurosporine or H-7 inhibited the stimulatory effect of NE (10^{-9} M) in a dose-dependent manner (Figs. 5 and 6). These studies indicate that the stimulatory effect of NE may be mediated via either the cAMP-dependent protein kinase A I and II or

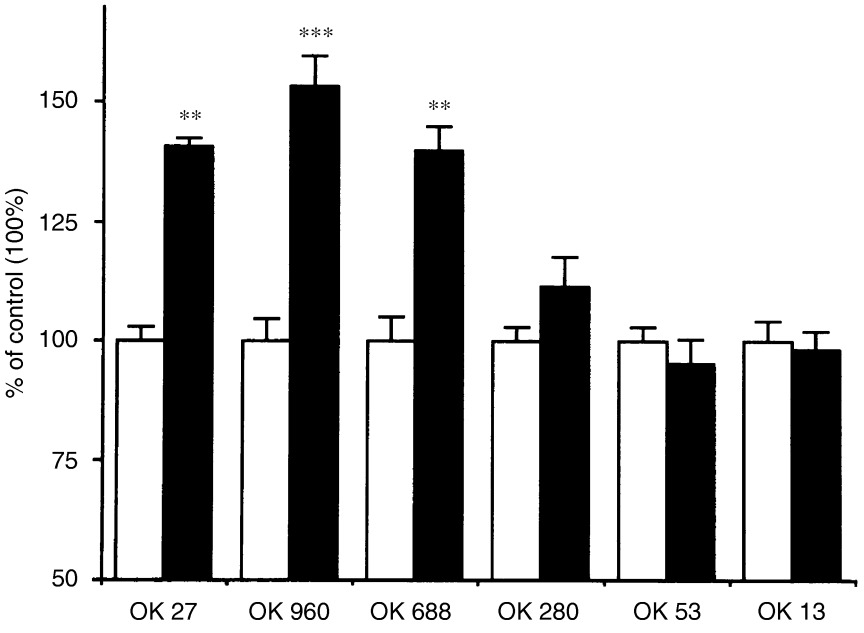

Fig. 8. Effect of norepinephrine on the expression of various ANG-GH fusion genes in OK cells. Cells were incubated for up to 24 hours in the presence of norepinephrine (10^{-9} M). Media were harvested after 24 hours of incubation and assayed for IR-hGH. The concentration of IR-hGH in the medium without norepinephrine (that is, OK 27 cells, 1.45 ± 0.03 ng/ml; OK 960, 1.29 ± 0.04 ng/ml; OK 688, 3.24 ± 0.2 ng/ml; OK 280, 0.37 ± 0.02 ng/ml; OK 53, 0.44 ± 0.02 ng/ml; OK 13, 5.56 ± 0.3 ng/ml) is considered as the control level. Each point represents the mean ± SD of three dishes (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from two other experiments.

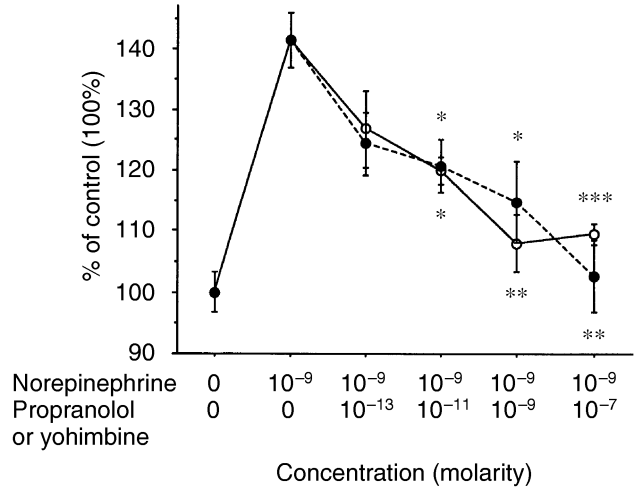

Fig. 9. Inhibitory effect of propranolol or yohimbine on the expression of pOGH (ANG N-806/-779/-53/+18) in OK 95 cells stimulated by norepinephrine (10^{-9} M). OK 95 cells were incubated for up to 24 hours in the presence of norepinephrine (10^{-9} M) and various concentrations (10^{-13} to 10^{-7} M) of propranolol or yohimbine. Media were harvested and assayed for the level of IR-hGH. The concentration of IR-hGH in the absence of norepinephrine or propranolol or yohimbine is the control level (that is, 3.25 ± 0.06 ng/ml). The inhibitory effect of propranolol or yohimbine was compared to cells which were incubated with 10^{-9} M norepinephrine. Results are expressed as the mean ± SD of three determinations (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Symbols are: (○) propranolol; (●) yohimbine. Similar results were obtained from two other experiments.

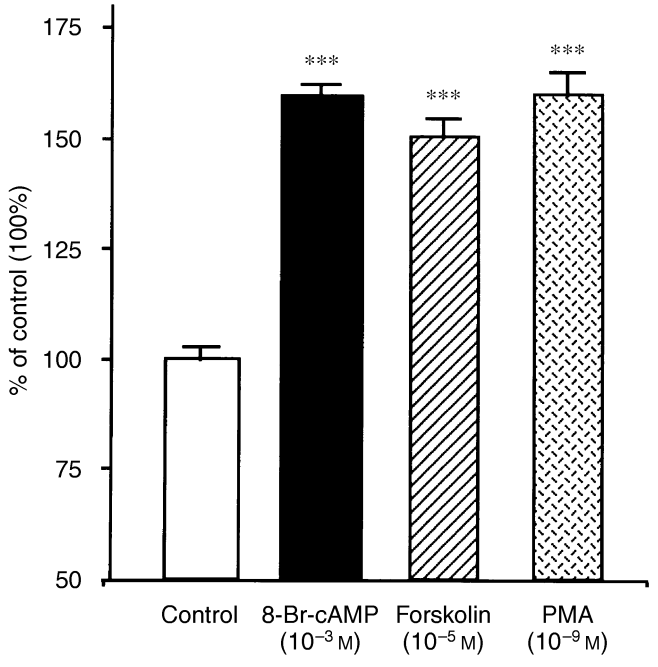

Fig. 10. Effect of 8-bromo-cAMP, forskolin and PMA on the expression of pOGH (ANG N-809/-779/-53/+18) in OK 95 cells. Cells were incubated for 24 hours in the presence of 10^{-3} M 8-Br-cAMP or 10^{-9} M forskolin or 10^{-9} M PMA. The levels of IR-hGH in the media were assayed by RIA-hGH. The concentration of IR-hGH in the absence of 8-bromo-cAMP, forskolin or PMA is the control (that is, 2.82 ± 0.12 ng/ml of IR-hGH). Each point represents the mean ± SD of three determinations (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.005$). Similar results were obtained from two other experiments.

PKC signal transduction pathways or via the interaction of both pathways in OK 27 cells. The involvement of the PKC pathway is further supported by the observation that U73122 inhibited the stimulatory effect of NE (Fig. 5). Since U73122 is an inhibitor of phospholipase C and phospholipase A_2, it is conceivable that the addition of U73122 might prevent the hydrolysis of phosphatidylinositol 4,5 biphosphate and would subsequently inhibit the activation of protein kinase C in OK 27 cells. Indeed, this possibility is supported by the studies of Martin et al, who showed that the addition of U73122 abolishes the increase

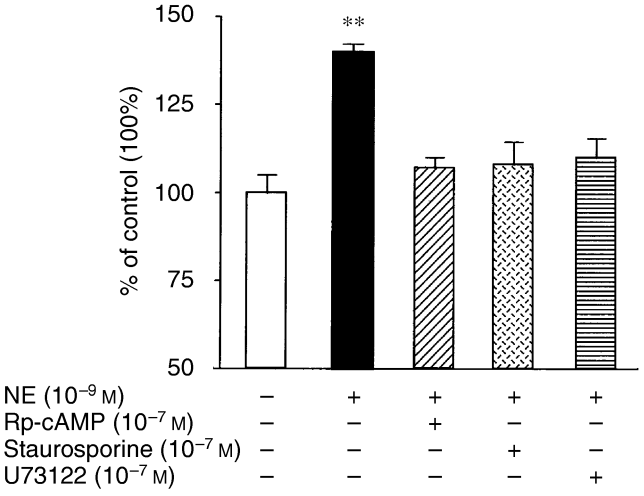

Fig. 11. Inhibitory effect of Rp-cAMP or U73122 or staurosporine on the expression of pOGH (ANG N-806/-779/-53/+18) in OK 95 cells stimulated by norepinephrine (10^{-9} M). OK 95 cells were incubated for up to 24 hours in the presence of norepinephrine (10^{-9} M) and 10^{-7} M Rp-cAMP or 10^{-7} M U73122 or 10^{-7} M staurosporine. Media were harvested and assayed for the level of IR-hGH. The concentration of IR-hGH in the absence of drugs is the control level (that is, 3.6 ± 0.03 ng/ml of IR-hGH). The inhibitory effect of Rp-cAMP or U73122 or staurosporine was compared to cells, which were incubated with 10^{-9} M norepinephrine. Results are expressed as the mean ± SD. of three determinations ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.005$). Similar results were obtained from two other experiments.

in particulate PKC activity stimulated by PTH in OK cells [29]. Our preliminary results also showed that the stimulatory effect of NE (10^{-9} M) and iodoclonidine (10^{-9} M) was abolished after a 24 hours pre-incubation of OK 27 cells with 10^{-5} M PMA compared to the control cells without the pre-incubation with PMA (unpublished results). These results are in agreement that the exposure to PMA will downregulate the PKC activity [30]. Moreover, our preliminary studies showed that NE at 10^{-9} M increased the cellular level of DAG and PKC activity in OK cells but not NE at 10^{-5} M (unpublished results). All these studies support the notion that the stimulatory effect of NE is mediated, at least in part, via the PKC signal transduction pathway.

At present, we do not have a good explanation for the results that the addition of 10^{-7} M atenolol, yohimbine, staurosporine, U73122 or H-7 alone completely inhibited the NE (10^{-9} M) stimulation of ANG gene expression (Figs. 3 to 6). One possible explanation may be that the dose 10^{-7} M was too high (100-fold excess), since equal molar concentration (10^{-9} M) of atenolol, yohimbine, staurosporine, U73122 or H-7 could only partially block the stimulatory effect of NE. The second possible explanation may be that there are multiple isoform(s) of PKC and adenylyl cyclase (AC) in OK cells. The effect of NE may be mediated via the interaction or "cross-talk" of these isoforms of PKC and AC. Indeed, studies have shown that PKC may interact with specific subtypes of AC but not others in a cell-specific manner [31]. Thus, it is possible that the addition of high levels (10^{-7} M) of atenolol, yohimbine, staurosporine, U73122 or H-7 might block the "cross-talk" between the PKC and PKA signal transduction pathway. Studies are underway in our laboratory to identify the isoform(s) of PKC and AC in OK cells.

Most interestingly, the stimulatory effect of the addition of a combination of both isoproterenol and iodoclonidine on the expression of the pOGH (ANG N-1498/+18) was significantly higher than the addition of isoproterenol and iodoclonidine alone ($P \leq 0.05$; Fig. 7), suggesting a synergistic effect of β- and α_2-adrenoceptors. Since the effect of β- and α_2-adrenoceptors are mediated via protein kinase A (PKA) and protein kinase C (PKC) in OK 27 cells, respectively [9, 10], these studies support the notion that there might be a "cross-talk" between the β- and α_2-adrenoceptors or between the activation of PKA and PKC on the expression of pOGH (ANG N-1498/+18) in OK 27 cells. These results are similar to our recent report [32] that the addition of a combination of SKF-82958 (a D_1-dopaminergic receptor agonist) and PPHT (a D_2-dopaminergic receptor agonist) significantly enhanced the expression of pOGH (Ang N-1498/+18) in OK 27 cells compared to the addition of SKF-82958 or PPHT alone. The effect of SKF-82958 and PPHT are mediated via the PKA and PKC signal transduction pathway, respectively [32]. At present, we do not understand the molecular mechanism(s) for the synergistic effect of β- and α_2-adrenoceptors in OK cells. Experiments are underway in our laboratory to explore the molecular mechanism(s) of the synergistic effect of β- and α_2-adrenoceptors on the expression of the ANG gene in OK cells.

Our present results showed that the addition of NE (10^{-9} M) stimulates the expression of pOGH (ANG N-1498/+18), pOGH (ANG N-960/+18) and pOGH (ANG N-688/+18) in OK 27, OK 960 and OK 688 cells, respectively. The addition of NE (10^{-9} M), however, had no effect on the expression of pOGH (ANG N-280/+18), pOGH (ANG N-53/+18) and pTKGH in OK 280, OK 53 and OK 13 cells, respectively (Fig. 8). These studies indicate that the NE-responsive element is probably localized within nucleotides N-1498 to N-280 in the 5′-flanking region of the rat ANG gene. Indeed, this possibility is supported by those studies (Fig. 9) that the addition of NE (10^{-9} M) stimulated the expression of the pOGH (ANG N-806/-779/-53/+18) in OK 95 cells. The DNA fragment, ANG N-806/-779 contains a CRE-motif (that is, N-795 to N-788, TGACGTAC), which is almost identical to the consensus CRE-motif of the somatostatin gene (that is, TGACGTCA) [33]. Moreover, the stimulatory effect of NE was inhibited in the presence of propranolol or yohimbine (Fig. 9). These studies demonstrate that the NE-responsive element is probably localized in the DNA fragment, ANG N-806/-779 of the rat ANG gene.

On the other hand, we were surprised that the addition

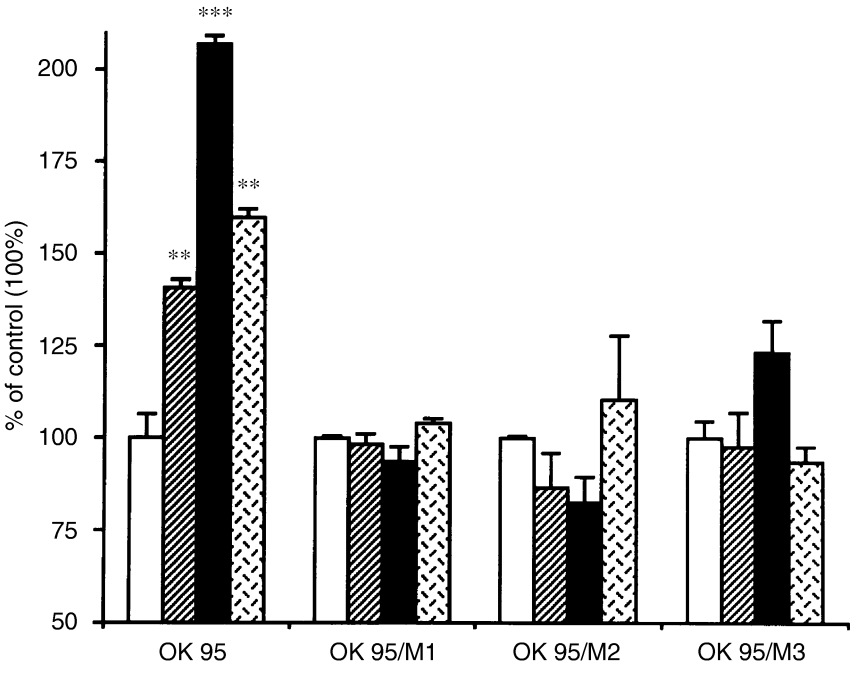

Fig. 12. Effect of norepinephrine, 8-bromo-cAMP and PMA on the expression of pOGH (AnG N-806/-779/-53/+18) and its mutants in OK 95, OK 95/M1, OK 95/M2 and OK 95/M3 cells. Cells were incubated for up to 24 hours in the presence of norepinephrine (10^{-9} M) or PMA (10^{-7} M) or 8-bromo-cAMP (10^{-3} M). Media were harvested after 24 hours of incubation and assayed for IR-hGH. The concentration of IR-hGH in the absence of norepinephrine or 8-bromo-cAMP or PMA represents the control level (that is, OK 95, 3.8 ± 0.2 ng/ml; OK 95/M1, 3.9 ± 0.01 ng/ml; OK 95/M2, 4.2 ± 0.05 ng/ml; OK 95/M3, 3.1 ± 0.14 ng/ml of IR-hGH). Each point represents the mean ± SD of three determinations ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.005$). Symbols are: (□) the control level; (▨) incubation medium in the presence of 10^{-9} M NE; (■) incubation medium in the presence of 10^{-7} M PMA; (▩) medium in the presence of 10^{-3} M 8-bromo-cAMP. Similar results were obtained from four other experiments.

of NE stimulated the expression of pOGH (ANG N-688/+18) in OK 688 cells. We did not find any consensus CRE motif (that is, TGACGTCA) in the promoter region between N-688 to N+281. These observations suggest that there might be an additional functional CRE-like in the region between N-688 to N-281. Studies are underway in our laboratory to identify the putative CRE-like in this region.

The addition of 8-bromo-cAMP, forskolin or PMA alone also stimulated the expression of the pOGH (ANG N-806/-779/-53/+18) in OK 95 cells compared to the control (without the addition of 8-bromo-cAMP, forskolin or PMA; Fig. 10). Furthermore, the stimulatory effect of NE on the expression of the pOGH (ANG N-806/-779/-53/+18) in OK 95 cells was inhibited in the presence of Rp-cAMP, staurosporine or U73122 (Fig. 11). These studies provide further support for the notion that the DNA fragment, ANG N-806/-779 is the CRE of the rat ANG gene.

Most convincingly, the addition of NE, forskolin or PMA had no stimulatory effect on the expression of mutants of the pOGH (ANG N-806/-779/-53/+18) in OK 95/M1, OK 95/M2 and OK 95/M3 cells (Fig. 12), these studies demonstrate that the DNA fragment, ANG N-806/-779 is the CRE of the rat ANG gene.

At present, we do not know the exact molecular mechanism(s) for the stimulatory effect of NE (that is, the downstream pathway after the activation of PKA and PKC) on the expression of pOGH (ANG N-1498/+18) in OK 27 cells. One possible explanation may be that NE might induce the phosphorylation of the nuclear cAMP-responsive element binding protein (CREB) at serine 133 via either PKA or PKC or a combination of both pathways, since studies have shown that CREB can be phosphorylated at serine 133 by cAMP-dependent protein kinase A [34], or by PKC [35, 36] and phosphorylation increases the binding affinity of 43 kD-CREB to CRE [35, 36] and subsequently enhances the gene expression. This possibility is supported by our most recent studies which showed that the addition of isoproterenol enhances the stimulating effect of 43 kD-CREB on the expression of the pOGH (ANG N-1498/+18) in OK 27 cells [38]. Indeed, more studies are ongoing to explore the molecular mechanism(s) of the stimulatory effect of NE on the expression of the ANG gene in OK cells.

Finally, it is evident that neither NE, forskolin, 8-Br-cAMP nor PMA had any effect on the expression of pOGH (ANG N-53/+18) in OK 53 cells (Fig. 13). Since the expression of the pOGH (ANG N-53/+18) is driven by the minimal promoter (that is, ANG N-53 to +18 contains the putative "CCAT" and "TATA" boxes on nucleotides-50 and -30 upstream of the transcriptional site, respectively [21]) of the rat ANG gene, these studies demonstrate that the minimal promoter of the rat ANG gene was not sufficient to respond to the addition of NE, forskolin, 8-Br-cAMP or PMA. The effect of NE, forskolin, 8-Br-cAMP and PMA in OK 27 is mediated via the putative CRE (ANG N-806 to N-779) in the 5′-flanking region of the rat ANG gene.

In summary, the present studies demonstrate that NE stimulated the expression of pOGH (ANG N-1498/+18) in OK 27 cells. The stimulatory effect of NE was blocked by the presence of propranolol or yohimbine or Rp-cAMP or staurosporine or U73122. Furthermore, we demonstrate

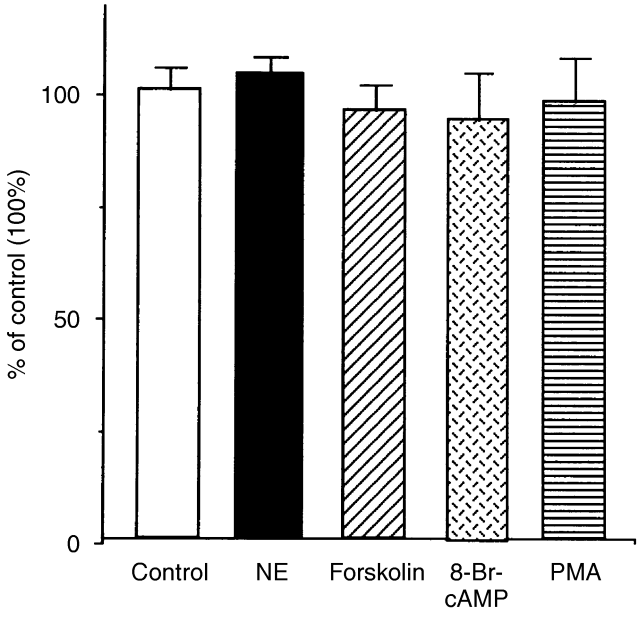

Fig. 13. Effect of norepinephrine or forskolin or 8-bromo-cAMP or PMA on the expression of pOGH (ANG N-53/+18) in OK 53 cells. Cells were incubated for 24 hours in the absence or presence of norepinephrine (10^{-9} M) or forskolin (10^{-7} M) or 8-Br-cAMP (10^{-3} M) or PMA (10^{-9} M). Media were harvested 24 hours after the incubation and assayed for IR-hGH. Each point represents the mean ± SD of three determinations. The concentration of IR-hGH in the absence of norepinephrine, forskolin, 8-Br-cAMP and PMA is the control level (that is, 2.57 ± 0.09 ng/ml of IR-hGH; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.005$). Similar experiments were obtained from two other experiments ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.005$).

that the effect of a combination of isoproterenol and iodoclonidine on the expression of the pOGH (ANG N-1498/+18) in OK 27 cells was significantly higher when compared to the addition of isoproterenol or iodoclonidine alone. Finally, we demonstrate that the stimulatory effect of NE is mediated via the putative cAMP-responsive element (CRE) in the 5′-flanking region of the rat ANG gene. Our studies suggest that the activation of renal nerves will release NE, which then stimulates the expression of the ANG gene in the renal proximal tubule and increases the formation of local renal Ang II. The elevated renal Ang II subsequently modulates the sodium and fluid reabsorption by the proximal tubular cells. Hence, the local intrarenal RAS plays a significant role in the modulation of sodium reabsorption.

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